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Ultrastructural Observations on Sperm Penetration in the Egg of Elkhorn Sculpin, *Alcichthys alcicornis*, Showing Internal Gametic Association

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ABSTRACT—Early fertilization process has been investigated ultrastructurally in elkhorn sculpin, *Alcichthys alcicornis*. In the eggs before immersion in sea water, spermatozoon was seen to be attached to the ooplasmic membrane at the site of an inner opening of the micropylar canal. By 10 sec after immersion in sea water, a fertilizing spermatozoon penetrated the ooplasm for about half the head length, and vesiculation occurred on the fused plasma membrane at the apical region of sperm head. By 30 sec after the immersion, the fertilizing spermatozoon showed a change in the tail axoneme, indicating a loss of its motility. By 1–2 min after the immersion, spermatozoon further penetrated the ooplasm up to its middle piece. It is worthy to note that a portion of ooplasm containing no significant cytoplasmic organelles other than ribosomes appeared around the penetrated sperm head and came to expand during the sperm penetration to form a triangular region surrounding the sperm head. This ooplasmic region may have a role in an active engulfment of immotile spermatozoon into the ooplasm. By 3 min after the immersion, sperm nuclear membrane was separated from chromatin and became vesiculated starting from the anterior region of sperm head caudalwards. Details of the events in early fertilization process were presented.

INTRODUCTION

An extensive work on the ultrastructural level has been accumulated dealing with early events occurring in the course of fertilization in teleosts. Initial stages of sperm-egg fusion and the formation of fertilization cone have been described in mummichog, *Fundulus heteroclitus* [2], and common carp, *Cyprinus carpio* [8]; the process of sperm penetration has been traced, though partially, in several species of teleosts [3, 7, 10, 13, 17, 18, 22, 24]; and changes of sperm nuclear membrane and the formation of the second polar body and blastodisc have been observed in rose bitterling, *Rhodeus ocellatus ocellatus* [18] and the mummichog [3], respectively. However, few studies have so far examined a complete spectrum of these continuous events of early fertilization in any single species of fishes. This seems to be due mainly to a difficulty of determining an actual time of occurrence of these successive events, since these events may proceed rather asynchronously in a mass of eggs after insemination. Only the work of Iwamatsu and Ohta [6] has revealed, using denuded and polyspermic eggs of medaka, *Oryzias latipes*, a series of ultrastructural changes from an initial stages of sperm-egg fusion to the formation of male pronucleus.

Elkhorn sculpin, *Alcichthys alcicornis*, has a unique mode of reproduction called "internal gametic association" [14]. In this teleost species, sperm is introduced into ovarian cavity by copulation and enters the micropylar canal of eggs ovulated into the ovarian cavity, but actual sperm-egg fusion does not occur within the ovary until the eggs have been released into sea water. In eggs freshly stripped from copulated females, sperm remains to be attached to the ooplasmic surface, and an actual fertilization reaction is initiated quite

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synchronously when the eggs are immersed in sea water. Availability of a lot of sperm-associated eggs from a single, copulated female allows samplings of these eggs in accurate time sequences after immersion of the eggs in sea water. Thus the eggs of the elkhorn sculpin appear to provide a useful model system for the time-course studies of early events occurring during fertilization reaction in teleost fishes. In the present study, ultrastructural features were described on gametes in the course of serial changes from "attachment" through "binding" to the early step of male pronucleus formation, with special reference to a characteristic ooplasmic movement having a possible relation with sperm penetration into the ooplasm.

MATERIALS AND METHODS

Adult males and females of the elkhorn sculpin used in the present study were captured by a trammel set along the shore of Usujiri, southern Hokkaido, during spawning season (March–April) in 1989–1991. Mature eggs and sperm were obtained by manually pressing the abdomen of mature fish. Spermatozoa, unfertilized eggs and fertilized eggs sampled at various intervals of time after immersion in sea water at 10°C were fixed with Karnovsky's fixative for 5 hr at room temperature. For transmission electron microscopy, after being washed with cold 0.1 M cacodylate buffer (pH 7.4), the specimens were post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer for 2 hr at 4°C. After dehydration through a graded ethanol series, the specimens were embedded in Epon. Ultrathin sections were prepared with a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate or with lead citrate alone, and examined with a Hitachi H-7000 electron microscope. Epon-embedded eggs were sectioned at about 1 μm in thickness, and stained with toluidine blue for light microscopy. For scanning electron microscopy, the fixed specimens were washed with 0.1 M cacodylate buffer and dehydrated in a graded ethanol series followed by isoamyl acetate. Then the specimens were dried with liquid CO_2 in a critical point drier. The dried specimens were coated with gold and examined with a Hitachi S-2300 electron microscope.

RESULTS

Sperm morphology

The mature spermatozoon of the elkhorn sculpin measures about 37 μm in total length. Its head is of thick spatula-shape, being about 2.9 μm long, 1.4 μm wide and 0.8 μm thick, and is devoid of acrosomal structure (Fig. 1). The sperm nucleus is composed of homogeneously condensed chromatin. On one side of the flattened nucleus, two centrioles are present slightly posterior to the middle of its length. From the distal centriole a groove with an axonema runs posteriorly along the

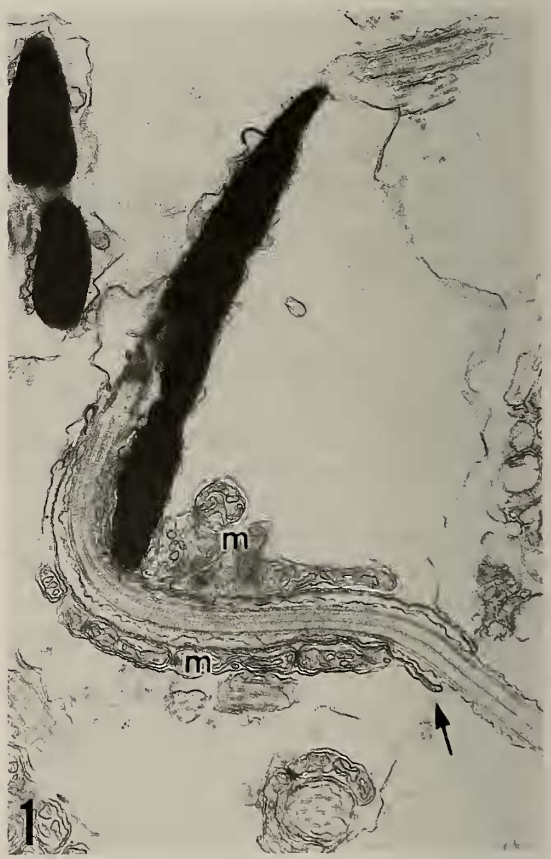

Fig. 1. TEM micrograph of a sagittal section of the sperm head and middle piece. Numerous mitochondria (m) are seen in the middle piece. Arrow indicats a cytoplasmic sleeve surrounding the proximal portion of the sperm tail. ×15,000.

long axis of the head. A middle piece conjoins the posterior end of the head, with many small-sized mitochondria aggregating compactly. It is noted that, on the centriolar side of the nucleus, several mitochondria extend their distribution anteriorly along the axonema in the head portion. A short, thin cytoplasmic sleeve surrounds the proximal portion of the sperm tail (Fig. 1 arrows). The flagellum has a typical 9+2 arrangement of microtubules.

Micropylar apparatus

The mature egg of elkhorn sculpin is globular in form measuring about 1.2 mm in diameter, and is covered with a vitelline envelope of about 40 μm thick. A micropylar apparatus is located at the animal pole of the egg. The micropyle is composed of a micropylar vestibule forming a shallow depression of about 70 μm in diameter on the chorion, and a micropylar canal which opens in the center of the micropylar vestibule (Fig. 2a). The micropylar canal is about 5.5 μm in diameter at its outer opening, and tapers gradually toward the inner opening of about 1.4 μm in diameter which allows only one spermatozoon to pass through (Fig. 2b). An ooplasmic projection containing a few ribosomes exists just beneath the micropylar canal, and protrudes into the micropylar canal for about 0.6 μm (Fig. 2b).

Morphological changes of the egg after immersion in sea water

In the elkhorn sculpin, sperm is introduced by

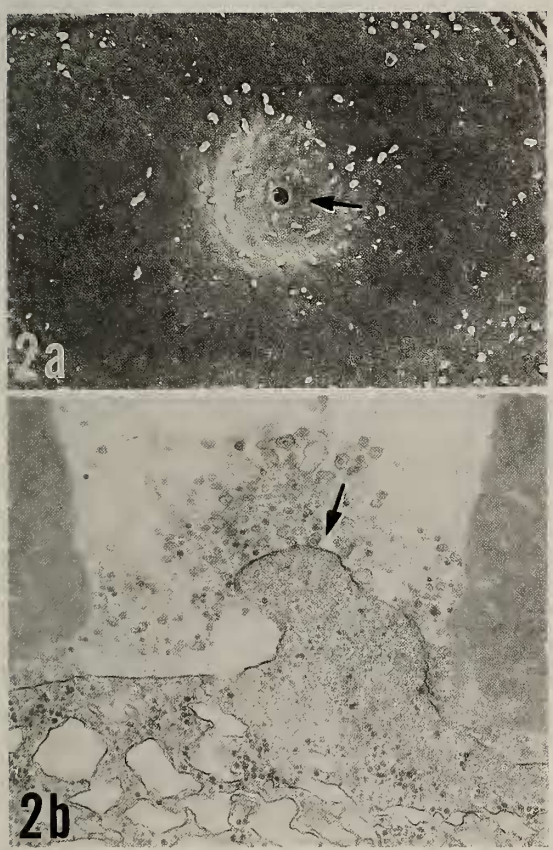

FIG. 2. (a), SEM micrograph of the animal pole of an egg, showing a micropylar apparatus consisting of a vestibule and a canal (arrow). ×285. (b), TEM micrograph of an inner opening of the micropylar canal. Arrow indicates an ooplasmic projection beneath the micropylar canal. ×30,000.

FIG. 3. TEM micrograph of the micropylar canal of an egg before immersion in sea water. A spermatozoon is attached to the ooplasmic projection, but a fusion of its plasma membrane with ooplasmic membrane has not yet occurred. ×20,000.

copulation into the ovarian cavity. In the eggs stripped from copulated females, many spermatozoa had entered the micropylar canal, and a single spermatozoon was seen to be attached to the plasma membrane of ooplasmic projection in the inner opening of the micropylar canal (Fig. 3). However, fusion between sperm and ooplasmic membrane had not yet occurred at this stage, since no spermatozoon remained on the ooplasmic surface around the inner opening of the micropylar canal after the removal of the vitelline envelope from freshly stripped eggs.

Eggs from copulated females were immersed into sea water immediately after stripping. Five sec after immersion, the spermatozoon attached to ooplasmic membrane did not show any change. It was remarked that the ooplasmic projection had disappeared around the attached spermatozoon which came to lie on the flat surface of ooplasm. Membrane fusion between spermatozoon and egg could not be observed yet at that time. Cortical alveoli were distributed closely abutting against the ooplasmic membrane, without showing an actual membrane fusion.

Membrane fusion between spermatozoon and egg had already completed 10–15 sec after immersion. Fertilizing spermatozoon penetrated the ooplasm for about half the head length (Fig. 4), and a vesiculation occurred on the plasma membrane at the apical region of sperm head fused to ooplasmic membrane. As the vesiculation advanced, the sperm plasma membrane disappeared completely to expose the sperm nuclear membrane to ooplasm. In the ooplasm around the penetrated sperm head, there were vesicular membranous structures which appeared to originate in the vesiculated plasma membrane of spermatozoon and egg (Fig. 4). Part of ooplasm came to protrude along the unpenetrated part of sperm head to form the so-called fertilization cone. Furthermore, an ooplasmic region containing no particular cytoplasmic organelles appeared surrounding the penetrating sperm head (Fig. 4). Membranes of cortical alveoli near the micropylar region were fused to the ooplasmic membrane, and the cortical alveoli were broken down to release their contents into the space between the vitelline envelope and ooplasmic membrane.

Fig. 4. Electron micrograph of the egg by 10 sec after immersion in sea water. An fertilizing spermatozoon penetrates the ooplasm and membrane fusion between spermatozoon and egg has begun. Several vesiclar structures are seen near the penetrating sperm head (arrows). ×20,000.

Twenty to thirty sec after immersion, the spermatozoon penetrated the ooplasm for more than half the head length. The membrane of sperm head had completely disappeared (Fig. 5). The ooplasm surrounding the penetrating spermatozoon contained few cytoplasmic organelles. At this stage, the sperm tail which remained outside of ooplasm was shown to be completely lacking in the typical 9+2 structure of its flagellar microtubles, and consisted of numerous fibrillar structures running through its length (Fig. 5). Such a structural change of flagellar microtubles seemed to accompany the progress of fertilization reaction in the penetrating spermatozoon. The breakdown of cortical alveoli was advancing further near the micropylar region, but not in other regions of the cortical cytoplasm of the egg.

FIG. 5. Electron micrograph of the fertilizing spermatozoon by 30 sec after immersion of the egg in sea water. The flagellar microtubles are degenerated. Sperm mitochondria (m) remain outside the egg. ×15,000.

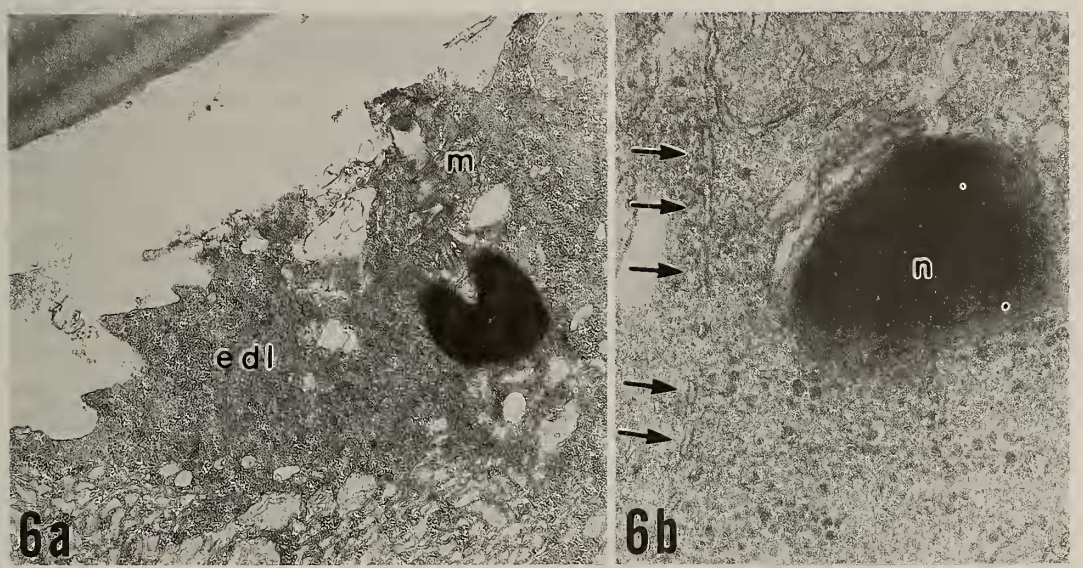

FIG. 6. TEM micrographs of eggs by 2 min after immersion in sea water. (a) Sperm head and mitochondria (m) have penetrated the ooplasm. The ooplasm surrounding the penetrating spermatozoon forms an electron dense, triangular zone (edl) containing lybosomes only. ×12,000. (b) The vesicular structures (arrows) are arranged in a row near the sperm nucleus (n). ×45,000.

One to two min after immersion, the spermatozoon penetrated the ooplasm further up to its middle piece. Sperm mitochondria, the basal part of axial filaments, and sperm nucleus covered with the nuclear membrane were all exposed to ooplasm (Fig. 6a). Several vesicles were seen to be arranged in a row in the ooplasm near the sperm head (Fig. 6b). A portion of the ooplasm containing only ribosomes as a significant cytoplasmic organelles formed a rather electron-dense, trian-

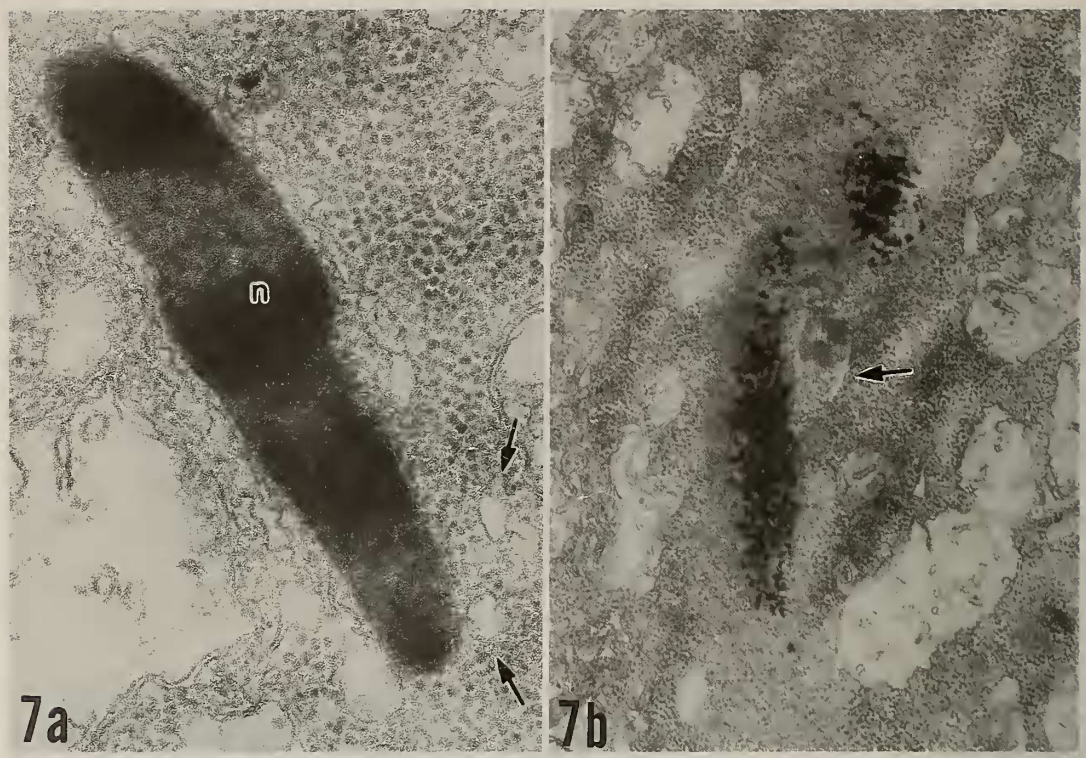

Fig. 7. Electron micrographs of eggs by 3 min after immersion in sea water. (a) The penetrated sperm nucleus (n) in the ooplasm. The nuclear membrane of spermatozoon becomes vesiculated begining from the anterior region of sperm head (arrows). ×60,000. (b) The chromatin of the penetrated sperm nucleus is dispersed. A fragment of nuclear membrane is visible near the centriol (arrow). ×60,000.

gular zone expanding from the apex of sperm head to the associated surface ooplasm (Fig. 6a).

Three min after immersion, the spermatozoon penetrated the ooplasm much deeper, and an electron dense layer was seen over the sperm head. The nuclear membrane of fertilizing spermatozoon was separated from the chromatin aggregation, and became vesiculated beginning from the anterior region of sperm head (Fig. 7a). In that region the chromatin began to disperse into ooplasm, forming a granular structure. In the eggs at this stage of fertilization, granular chromatin, centriole, and the basal part of axial filaments of spermatozoon were seen in the ooplasm. A single

Fig. 8. TEM micrograph of an egg by 5 min after immersion in sea water. The second polar body undergoing second meiotic division is seen in the perivitellin space. Numerous microtubles are present in the cytoplasmic bridge. ×4,000.

vesicle which seemed to be a part of vesiculated nuclear membrane was seen in ooplasm near the spermatozoan centriole (Fig. 7b).

Five min after immersion, fertilizing spermatozoon could not be discerned in ooplasm even by electron microscopy. A second polar body undergoing second meiotic division was seen in the perivitelline space near the micropylar region (Fig. 8). The second polar body was connected with ooplasm by a cytoplasmic bridge in which a number of microtubles ran vertically. The surface of the second polar body was complicatedly crumpled.

DISCUSSION

Fertilization is defined as the fusion of a male and a female gamete followed by nuclear fusion between the two gametes [1]. In the present study on the elkhorn sculpin, it was confirmed that spermatozoa entered the micropylar canal of ovulated eggs and were attached to the ooplasmic membrane without showing any membrane fusion between spermatozoon and egg until after the egg had been released into sea water. It is of significance to refer to such a peculiar reproductive mode as "internal gametic association" in order to distinguish it from the actual oviparity [14].

Spermatozoa of teleostean fishes are different in shape in different species, though they lack an acrosomal structure without exception. Spermatozoon of the elkhorn sculpin has a relatively long middle piece and a long, flattened head. Spermatozoa of a similar shape were reported to occur also in *Oligocottus maculosus* [20], *Cottus hangiongensis* and *C. nozawae* [19], suggesting that such a shape of spermatozoa may be common to fishes belonging to the family Cottidae. However, the spermatozoa of the elkhorn sculpin have a larger number of mitochondria as compared with those of other cottid species. Such a feature may reflect the fact that the spermatozoa of the sculpin can be motile for a very long time in ovarian fluid of the same species (data not shown).

It has been shown in many teleosts that there is a specialized conformation to attach the spermatozoon to the surface of ooplasm beneath the micropylar canal. Kudo [9] showed that the conformation in common carp, Japanese dace, *Tribolodon hakonensis,* and ayu, *Plecoglossus altivelis* was an ooplasmic projection extending up into the micropylar canal and had a function as a receptor for fertilizing spermatozoon. The existence of such a conformation has been demonstrated also in mummichog [2], chum salmon, *Oncorhynchus keta* [12], rose bitterling [18], and zebrafish, *Brachydanio rerio* [5]. The egg of the elkhorn sculpin also had a cytoplasmic protrusion on the egg surface beneath the micropylar canal, and a spermatozoon came to attach to this protrusion after passing through the canal. In the elkhorn sculpin, however, such cytoplasmic protrusion was observed to exist not only just beneath the micropylar canal but in other regions of ooplasmic surface near the micropyle. The present study could not confirm whether the cytoplasmic protrusion has a function of sperm receptor or not. It has been reported that polyslermy occurs in the medaka [6], and the rose bittering [15] when the eggs have been denuded mechanically. If the eggs of the elkhorn sculpin have a similar nature to those of the medaka and rose bitterling, it seems likely that the cytoplasmic protrusion beneath the micropylar canal does not have any special function for fertilization.

In the eggs stripped freshly from copulated female elkhorn sculpin, many spermatozoa have entered the micropylar canal and a spermatozoon have been attached to the egg surface, but the membrane fusion between spermatozoon and egg does not occur yet. The spermatozoon did not remain to be attached to the egg surface after removal of the vitelline envelope when observed by a scanning electron microscope. This indicates that the attachment between egg and spermatozoon in the ovarian lumen is relatively loose. In mammals the egg-sperm association includes two successive steps which are referred to as attachment and binding [21]. The attachment is a relatively loose, nonspecific association, while the binding is a tenacious, species-specific association [21]. Therefore the attachment of spermatozoon and egg in the ovary of the elkhorn sculpin may correspond to the nonspecific attachment of the gametes in mammals.

Membrane fusion between spermatozoon and egg has been studied in various species of animals.

It is known that the inner acrosomal membrane fuses with ooplasmic membrane in several marine invertebrates [4]. On the other hand, in mammals, a fusion between the plasma membranes of sperm head and egg microvilli occurs at the post-nuclear cap region of spermatozoon after the acrosomal reaction has completed [25]. Ultrastructural observations on membrane fusion between spermatozoon and egg in teleosts which is lacking in an acrosome on sperm head have been carried out in the medaka [6], rose bitterling [15, 17, 18], mummichog [3], common carp [11], and zebrafish [22]. However, information about gamete membrane fusion in teleosts has been relatively fragmentary as compared with that in other animal groups, possibly because of the fact that the membrane fusion between spermatozoon and egg in teleosts occurs in a short time after gamete association. As mentioned before, in the case of denuded eggs of the medaka [6], the ooplasmic membrane comes to fuse with the sperm plasma membrane in various regions one min after insemination, giving rise to polyspermy. Ohta [17] demonstrated that, in the rose bitterling, the fusion occurred between the microvillus membrane of a sperm entry site on the egg surface and the sperm head membrane at the portion in front of the centrioles. In the elkhorn sculpin, membrane fusion between spermatozoon and egg began by 10 sec after immersion in sea water. The membrane fusion occurred with a vesiculation of apical membrane of the sperm head and the ooplasmic membrane, and the vesiculation extended successively to the whole surface of the penetrated sperm head, regardless of the location of centrioles existing in the middle region of sperm head. Therefore, the membrane fusion in the elkhorn sculpin seems to occur in a similar manner to that in the medaka, and begins at the portion having no direct relation to the position of sperm centrioles.

By 30 sec after immersion in sea water, a typical 9+2 axonemal structure of the tail of fertilizing spermatozoon had disappeared. Since the microtubles of sperm tail are indispensable for sperm motility, such a change may indicate that the spermatozoon has lost it motility and the sperm tail is undergoing degeneration by this time. The ooplasm surrounding the penetrating spermatozoon was initially lacking in significant cytoplasmic organelles exhibited a relatively uniform structure. As the spermatozoon penetration proceeded, this part of ooplasm continued to expand inside to form a triangular region with accumulating ribosomes. Moreover, several vesicles, which were formed possibly by vesiculations of spermatozoon and ooplasmic membrane, were seen to be arranged in a row near the deeply penetrated sperm head, suggesting that the ooplasm was acting to draw the fertilizing spermatozoon inside into the egg. Such a change of ooplasm was never observed in unfertilized eggs even when they were immersed in sea water. These findings indicate that the ooplasm surrounding a fertilizing spermatozoon may have a role in active engulfment of the spermatozoon which has lost its motility by degeneration of tail axoneme. Wolenski and Hart [23] showed that the treatment of zebrafish eggs with cytochalasin B or D prevented the incorporation of fertilizing spermatozoon into eggs, and suggested the presence of actin-containing filaments in ooplasm which might play a role in the sperm incorporation. In the present study, although the precise mechanism of ooplasmic movement during penetration was not clarified, it is quite possible that ooplasmic microfilaments may be involved in the observed ooplasmic movement.

In medaka egg, sperm nuclear membrane is broken down by vesiculation along the lateral aspect of sperm nucleus by 3 min after insemination [6], while in rose bitterling egg, sperm nuclear membrane disappears first at the apical region of the head by 5 min after insemination [18]. In elkhorn sculpin egg, vesiculation of the outer and inner nuclear membrane occurs by 3 min after immersion of the egg in sea water. The vesiculation of sperm nuclear membrane started at the anterior region of sperm head and finished at the region near the centriole, like in the case of the medaka and the rose bitterling.

The process of formation of the second polar body in teleostean egg has been described in artificially activated eggs of the rose bitterling [16]. In this fish, the second polar body begins to rise by 15 min after activation, and completes its separation from the egg proper by 30 min later. In the

present study, a separating polar body was noticeable ultrastructurally 5 min after immersion of the eggs in sea water. The process of formation of the second polar body was confirmed to be generally similar to that found in the rose bitterling [16].

In present study, we clarified in detail the ultrastructural change of the elkhorn sculpin gametes in early fertilization processes. Moreover, we could suggest an ooplasmic movement for sperm penetration and the destiny of sperm tail during sperm penetration. Information obtained by the present study may be important to solve problems concerning the whole process of fertilization in teleosts.

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